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Long-chain polyunsaturated fatty acids are consumed during allergic inflammation and affect T helper type 1 (Th1)- and Th2-mediated hypersensitivity differently

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Summary

Studies have shown that atopic individuals have decreased serum levels of n-3 fatty acids. Indicating these compounds may have a protective effect against allergic reaction and/or are consumed during inflammation. This study investigated whether fish (n-3) or sunflower (n-6) oil supplementation affected T helper type 1 (Th1)- and Th2-mediated hypersensitivity in the skin and airways, respectively, and whether the fatty acid serum profile changed during the inflammatory response. Mice were fed regular chow, chow + 10% fish oil or chow + 10% sunflower oil. Mice were immunized with ovalbumin (OVA) resolved in Th1 or Th2 adjuvant. For Th1 hypersensitivity, mice were challenged with OVA in the footpad. Footpad swelling, OVA-induced lymphocyte proliferation and cytokine production in the draining lymph node were evaluated. In the airway hypersensitivity model (Th2), mice were challenged intranasally with OVA and the resulting serum immunoglobulin (Ig)E and eosinophilic lung infiltration were measured. In the Th1 model, OVA-specific T cells proliferated less and produced less interferon (IFN)-γ, tumour necrosis factor (TNF) and interleukin (IL)-6 in fish oil-fed mice *versus* controls. Footpad swelling was reduced marginally. In contrast, mice fed fish oil in the Th2 model produced more OVA-specific IgE and had slightly higher proportions of eosinophils in lung infiltrate. A significant fall in serum levels of long-chain n-3 fatty acids accompanied challenge and Th2-mediated inflammation in Th2 model. Fish oil supplementation affects Th1 and Th2 immune responses conversely; significant consumption of n-3 fatty acids occurs during Th2-driven inflammation. The latter observation may explain the association between Th2-mediated inflammation and low serum levels of n-3 fatty acids.

Keywords: allergy, atopic eczema, delayed-type hypersensitivity, fish oil, n-3 polyunsaturated fatty acids

Introduction

Several studies have shown a lower rate of atopic eczema in children whose diet has included fish [1–3]. Atopic eczema is defined as itchy skin lesions at typical locations, e.g. skin creases, as well as on the face and limbs in children younger than 4 years [4]. Atopic eczema is linked strongly to a history of asthma, hay fever and immunoglobulin (Ig)E-mediated food allergy in the individual and their family [5]. However, whereas asthma and hay fever are regarded as typical T helper type 2 (Th2)-driven inflammatory conditions, the pathogenesis of atopic eczema is more complex. In early lesions, skin-infiltrating T cells produce typical Th2 cytokines, such as interferon (IL)-4, while later, the typical Th1 cytokine interferon (IFN)-γ dominates [6]. These observations indicate that in atopic eczema Th2 cells rapidly initiate short-lasting inflammation, but that Th1 cells are responsible for the chronic inflammatory reaction that results in actual skin lesions [7].

Fish contains high levels of the long-chain n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs have immunoregulatory properties, and several studies have demonstrated lower serum levels of long-chain n-3 PUFAs in patients with atopy *versus* unaffected individuals [8–10]. However, other studies have shown the opposite result [11,12]. An association between low serum PUFA levels and active allergy may indicate either a protective effect of n-3

fatty acids, or enhanced consumption of these fatty acids during inflammation, or a combination of the two mechanisms. Evidence supporting an enhanced consumption of long-chain n-3 PUFAs includes a study in which children with atopic eczema were found to have lower serum levels of EPA and DHA than non-atopic children, despite similar levels of fish consumption [2]. Results from intervention studies have been inconclusive [13–15].

Various animal models have been used to study the role of n-3 PUFAs in atopic inflammation. Yokoyama *et al*. [16] showed a reduced atopic asthma reaction in a mouse model after exposure to aerosolized DHA. Yoshino and Ellis [17] reported a tendency towards reduced cell-mediated hypersensitivity reactions in mice fed a fish oil-supplemented diet. However, neither study noted any effect on IgE production. Yet another study reported decreased secretion of Th1-type cytokines [IFN-γ and tumour necrosis factor (TNF)-α], but enhanced secretion of the Th2 cytokine IL-4, from splenocytes in mice fed a fish oil-enriched diet [18].

The present study was designed to investigate the hypothesis that intake of long-chain n-3 PUFAs would affect Th1- and Th2-mediated sensitization and/or inflammation differentially. The effects of fish oil (rich in n-3 PUFAs) and sunflower oil (rich in n-6 PUFAs) intake were studied in two mouse hypersensitivity models: Th1-driven delayed-type hypersensitivity (DTH) and Th2-driven IgE production and eosinophil-mediated airway inflammation. In addition, the effect of PUFA consumption on the fatty acid serum profile was evaluated by monitoring serum levels during the study.

Materials and methods

Animals and diets

Four-week-old male BALB/c mice (Scanbur AB, Sollentuna, Sweden) were provided with food and water *ad libitum*. The mice were fed with one of three diets. The control group received regular mouse chow containing 1 wt% soya oil (Lantmännen, Lidköping, Sweden). The fish oil group received regular chow supplemented with 10 wt% fish oil containing 0·28 g EPA/ml and 0·34 g DHA/ml (Möllers Tran natural; Peter Möller, Oslo, Norway). The sunflower oil group received regular chow supplemented with 10 wt% sunflower oil containing 0·54 g linoleic acid/ml (Coop Solrosolja; Coop Sweden, Solna, Sweden). Permission for the study was granted by the Regional Ethics Committee, University of Gothenburg (no. 408-2008), and the experiments were carried out according to the guidelines of the 'Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific purposes'.

DTH model

Th1-mediated hypersensitivity was tested in the DTH model summarized in Fig. 1a. After receiving the experimental or control diet for 21 days, the mice were anaesthetized briefly (Isofluran; Baxter Medical AB, Kista, Sweden) and then each hind leg was injected intramuscularly with 50 μg ovalbumin (OVA) in 50 μl of phosphate-buffered saline (PBS), emulsified in an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). On day 42, 100 μl of emulsion made up using Freund's incomplete adjuvant was injected subcutaneously at the root of the tail. This regimen stimulates the development of Th1-polarized immunity to OVA. On day 50, the mice were anaesthetized and challenged with 100 μg of OVA in 30 μl of PBS by footpad injection. The DTH reaction was assessed by measuring tissue swelling in the footpad after 24 h (i.e. on day 51) using a caliper; the mice were then killed. To measure sensitization, the popliteal lymph nodes were excised and single cells were prepared under aseptic conditions and suspended in Iscove's medium supplemented with 2 mM L-glutamine, 50 μM mercaptoethanol, 50 μg/ml gentamycin and 10% fetal calf serum (all from Sigma, Steinheim, Germany). Samples of 1×10^5 cells/well were transferred to 96-well microtitre plates and stimulated with 0·5 mg/ml OVA and incubated in 5% CO_2 at 37°C. After 2 days, the supernatant was collected for cytokine analysis. After 7 days, [^{3}H]-thymidine was added and cells were harvested 10 h later. Cell proliferation was assessed by measuring [^{3}H]-thymidine incorporation in a β-counter (Perkin Elmer, Waltham, MA, USA).

Levels of IFN-γ, TNF and IL-6 in 2 days' supernatants were assayed by cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA) according to the manufacturer's recommendations. Samples were assayed using fluorescence activated cell sorter (FACS)Canto (BD Biosciences Pharmingen, San Jose, CA, USA) and analysed with FCAP Array Software (BD Biosciences). The limits of detection were 17·5 pg/ml for IFN-γ, 7·3 pg/ml for TNF and 5 pg/ml for IL-6.

Airway hypersensitivity model

The effects of the three different diets were also evaluated in a Th2-driven airway hypersensitivity model (Fig. 1b) in a second set of animals. Mice were immunized on days 15 and 25 with intraperitoneal (i.p.) injections of 10 μg OVA absorbed onto 2 mg of $Al(OH)_3$ (alum; Sigma). On day 33, the animals were anaesthetized briefly (Isofluran; Baxter Medical AB) and challenged with 100 μg of OVA in 25 μl of PBS by intranasal administration. This procedure was repeated on each of the following 4 days. Twenty-four hours after the final challenge, the mice were anaesthetized (xylazine 130 mg/kg and ketamine 670 mg/kg, i.p.). The chest was opened and blood was drawn by heart puncture. The blood sample was clotted and serum was collected after centrifugation (15 min at 3000 ***g***). Bronchoalveolar lavage was performed by twice instilling 0·4 ml of PBS through the trachea followed by gentle aspiration. The proportion of eosinophils in the bronchoalveolar fluid was evaluated on slides prepared using a cytospin and stained with May–Grünwald/Giemsa.

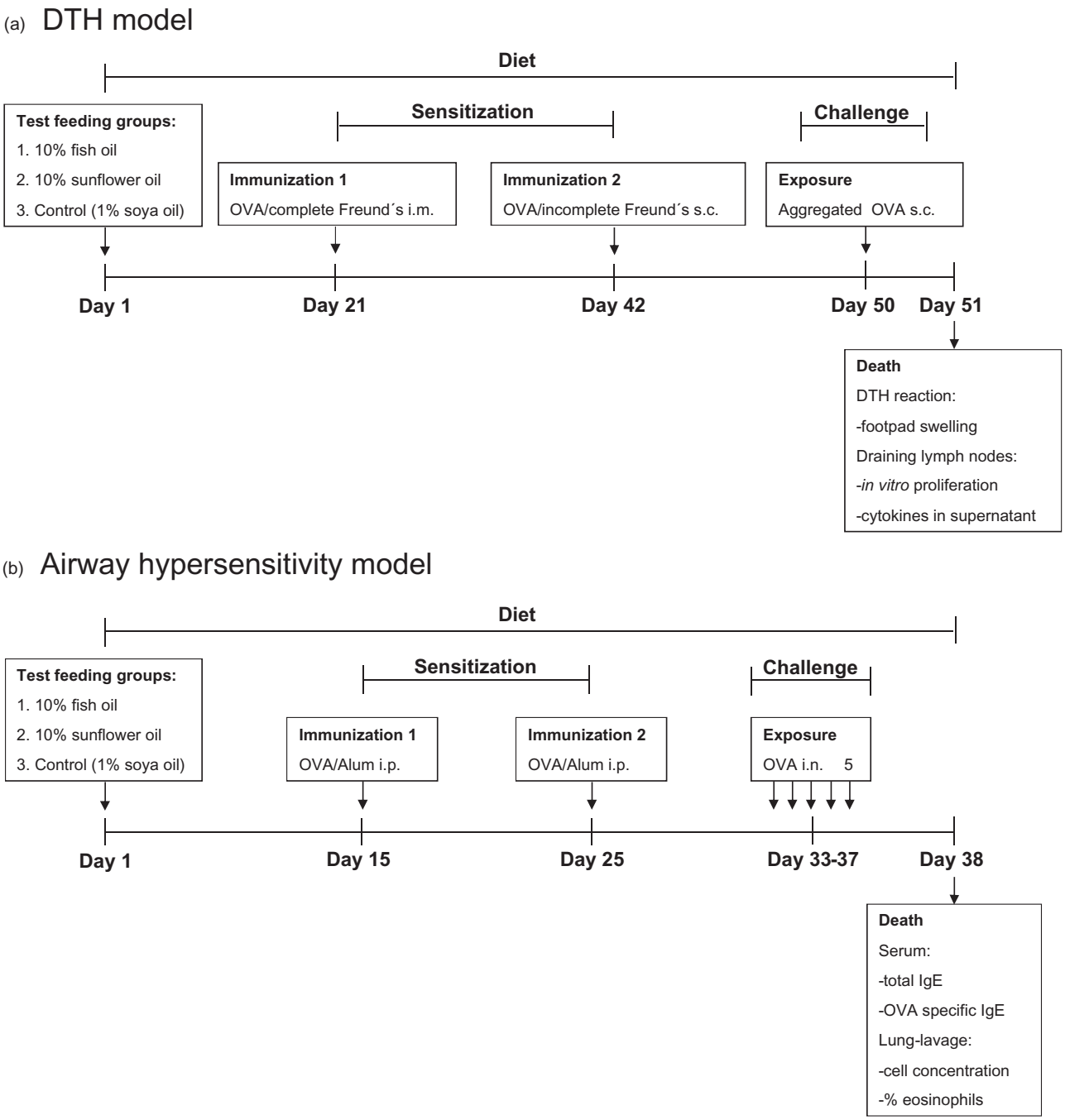

Fig. 1. Experimental design for the (a) delayed-type hypersensitivity (DTH) model [T helper type 1 (Th1)-mediated] and (b) airway hypersensitivity model (Th2-mediated). i.m., intramuscularly; i.n., intranasally; i.p., intraperitoneally; OVA, ovalbumin; s.c., subcutaneously.

Sensitization was measured as OVA-specific IgE titres in the serum samples by passive cutaneous anaphylaxis [19]. Mouse sera were diluted serially with PBS and 50 µl was injected intradermally into the shaved dorsal skin of anaesthetized (8 mg/kg xylazine and 40 mg/kg ketamine i.p.) Sprague–Dawley rats (Scanbur AB). After 3 days, the rats received an intravenous injection of 5 mg of OVA in 1 ml of PBS with 1% Evan's blue (Sigma); they were killed 30 min later. OVA-specific IgE titres were defined as the reciprocal of the highest dilution of serum giving a spot of ≥ 5 mm in diameter on the dorsal skin. Total serum IgE concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA). Costar plates were coated with 1 µg/ml mouse anti-IgE antibody; 2 µg/ml biotinylated anti-mouse IgE was used as the detection antibody and purified mouse IgE as the standard (all from BD Biosciences Pharmingen). The limit of detection was 6 ng/ml.

Analysis of fatty acids in mouse serum

In both experimental models, the fatty acid profile was monitored over time in serum samples collected before the start of the intervention and on three occasions during the study feeding period (days 25, 49 and 51 in the DTH model and days 14, 29 and 39 in the airway hypersensitivity model). Fatty acid (EPA, DHA and arachidonic acid) levels at each time-point were analysed by gas chromatography after conversion to methyl esters [20]. Mouse serum samples (100 µl) were mixed with 2 ml of toluene, 2 ml of acetyl chloride (10%) dissolved in methanol and 50 µl of internal standard (fatty acid 21:0, 0·5 mg/ml) and incubated in a waterbath at 70°C for 2 h. The methyl esters were extracted with petroleum ether; after evaporation, they were dissolved in iso-octane, separated by gas chromatography (Hewlett Packard 5890; Waldbronn, Germany) on an HP Ultra 1 (50 m × 0·32 mm × 0·52 µm DF) column (J&W Scientific, Folsom, CA, USA) and detected by flame ionization. Borwin software 1·21 (Le Fontanil, France) was used to analyse the chromatography data.

Statistical analysis

Mann–Whitney *U*-test was used to compare groups. Spearman's rank correlation was used to test for associations. Wilcoxon's signed-rank test was used to verify within-individual differences in serum fatty acids at the different time-points. Calculations were performed using SPSS version 15·0 (SPSS Inc., Chicago, IL, USA).

Results

Dietary effects on the DTH reaction (Th1 model)

In each of the two runs of this experiment, three groups of 12 mice received control, fish oil or sunflower oil diet. Mice fed fish oil supplemented diet displayed marginally but non-significantly less footpad swelling compared with the other two groups (Fig. 2a). In the sensitization test, lymphocytes from fish oil-fed mice showed significantly reduced OVA-induced proliferation compared with control ($P = 0{\cdot}004$) and sunflower oil ($P = 0{\cdot}01$)-fed animals (Fig. 2b). Analysis

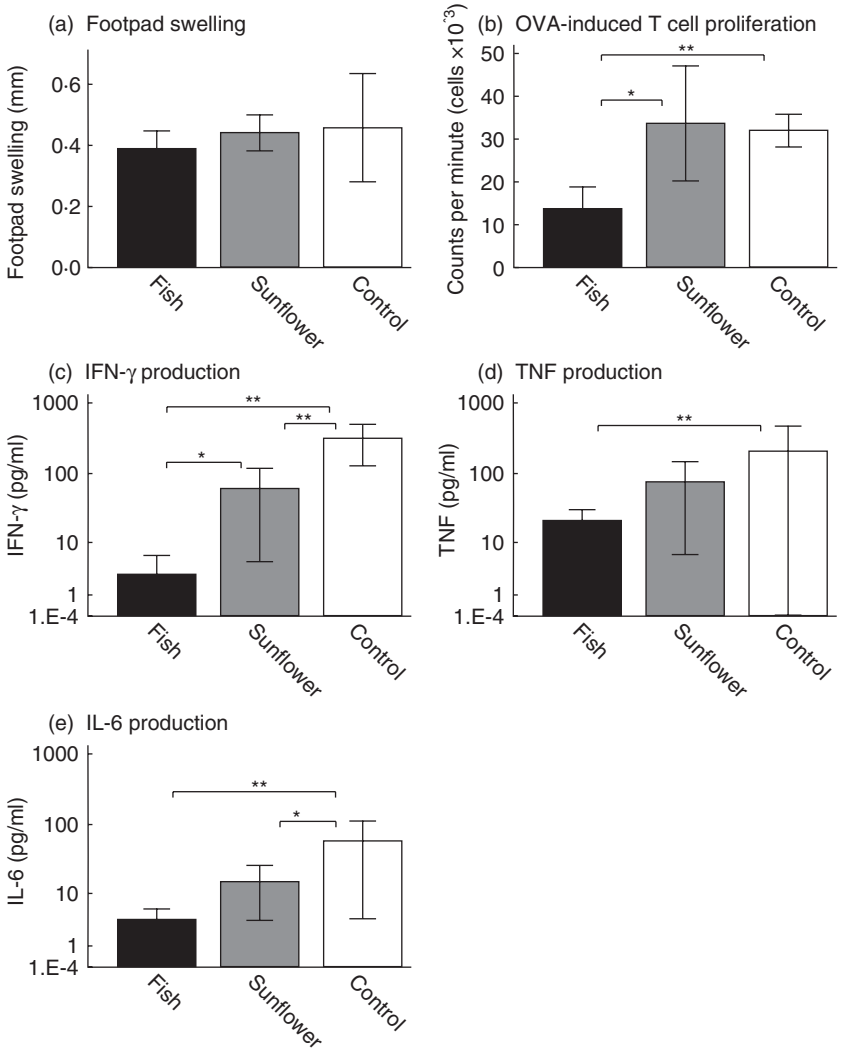

Fig. 2. Effects of fatty acid supplementation in the delayed-type hypersensitivity model: mice were fed fish or sunflower oil supplemented or control diets, immunized by ovalbumin (OVA) and challenged in the footpad. (a) Footpad swelling measured 24 h after challenge. (b) Proliferation of cells from draining lymph nodes and production of (c) interferon (IFN)-γ, (d) tumour necrosis factor (TNF) and (e) interleukin (IL)-6 after *in vitro* stimulation with OVA. The results are represented as mean ± standard error of the mean of 12 mice per diet group and are representative of two independent experiments (**$P < 0{\cdot}01$, *$P \leq 0{\cdot}05$).

of cytokines in the 2-day supernatants revealed significantly less production of the Th1 cytokine IFN-γ in fish oil-fed mice *versus* both control mice ($P = 0{\cdot}003$) and sunflower oil-fed mice ($P = 0{\cdot}02$) (Fig. 2c). Mice fed the sunflower oil diet also showed lower production of IFN-γ compared with control mice ($P = 0{\cdot}01$). The overall picture was the same for production of TNF (Fig. 2d) and IL-6 (Fig. 2e): fish oil-fed mice had significantly lower cytokine levels *versus* control mice (TNF; $P = 0{\cdot}004$, IL-6; $P = 0{\cdot}003$) and sunflower oil-fed mice produced lower IL-6 levels than control mice ($P = 0{\cdot}04$).

Dietary effects on the airway hypersensitivity (Th2) model

The effect of dietary fatty acids on Th2-driven sensitization and eosinophil-mediated inflammation was investigated in the airway hypersensitivity model. In each of the three runs of this experiment, three groups of seven mice received control, fish oil or sunflower oil diet. The proportion of eosinophils in the fluid tended to be higher in the fish oil group than in the control group ($P = 0{\cdot}05$) and in the sunflower group ($P = 0{\cdot}06$, Fig. 3a). The passive cutaneous anaphylaxis test showed that serum levels of OVA-specific IgE tended to be higher in the fish oil-fed mice, *versus* the sunflower oil-fed and control groups (both $P = 0{\cdot}06$, Fig. 3b). There was also a tendency for higher serum concentrations of total IgE in the fish oil-fed group ($P = 0{\cdot}09$ *versus* control mice; Fig. 3c).

Serum fatty acids

In the Th1 and Th2 models serum fatty acid levels were assessed before the dietary intervention, twice during the sensitization scheme and after the animals had been challenged with OVA in (i.e. when the inflammatory process was ongoing).

In fish oil-fed mice serum levels of EPA and DHA increased significantly during the first 3 weeks of the test diet (Fig. 4a,b), accompanied by an expected decrease in arachidonic acid. In sunflower oil-fed mice, arachidonic acid levels increased somewhat during the test diet feeding, with less effect on DHA and EPA (Fig. 4c,d). The third sample was drawn after two immunizations with OVA either in Freund's adjuvant (Th1 model) or alum (Th2 model). Interestingly, Th2 skewing immunization was accompanied by decreased levels of arachidonic acid, EPA and DHA in mice fed the sunflower oil and control diets (Fig. 4d,f). No such decreases accompanied Th1 immunization; indeed, DHA serum levels increased in control mice during the immunization phase (Fig. 4e).

The last sample was drawn after the challenge phase. Whereas challenge in the DTH (Th1) model had only small effects on the serum fatty acid profile (Fig. 4a,c,e), a significant drop in both EPA and DHA levels accompanied the challenge in the airway hypersensitivity model in fish oil-fed mice (Fig. 4b). There was also a non-significant drop in DHA in the sunflower oil-fed group (Fig. 4d) and in EPA

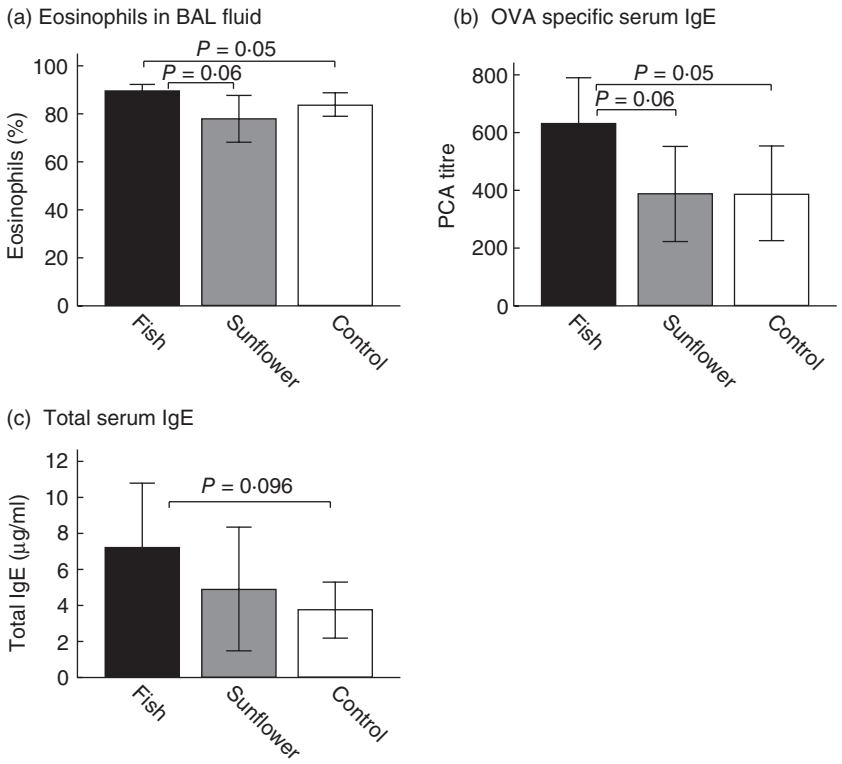

Fig. 3. Effects of fatty acid supplementation in the airway hypersensitivity model. Mice were fed fish ($n = 7$) or sunflower oil ($n = 7$) supplemented or control diet ($n = 7$), immunized and challenged intranasally with ovalbumin (OVA). Proportion of eosinophils (a) from bronchoalveolar lavage fluid. Serum levels of (b) OVA-specific immunoglobulin (Ig)E and (c) total IgE. BAL, bronchoalveolar lavage. The results are represented as mean ± standard error of the mean of seven mice per diet group and are representative of three independent experiments.

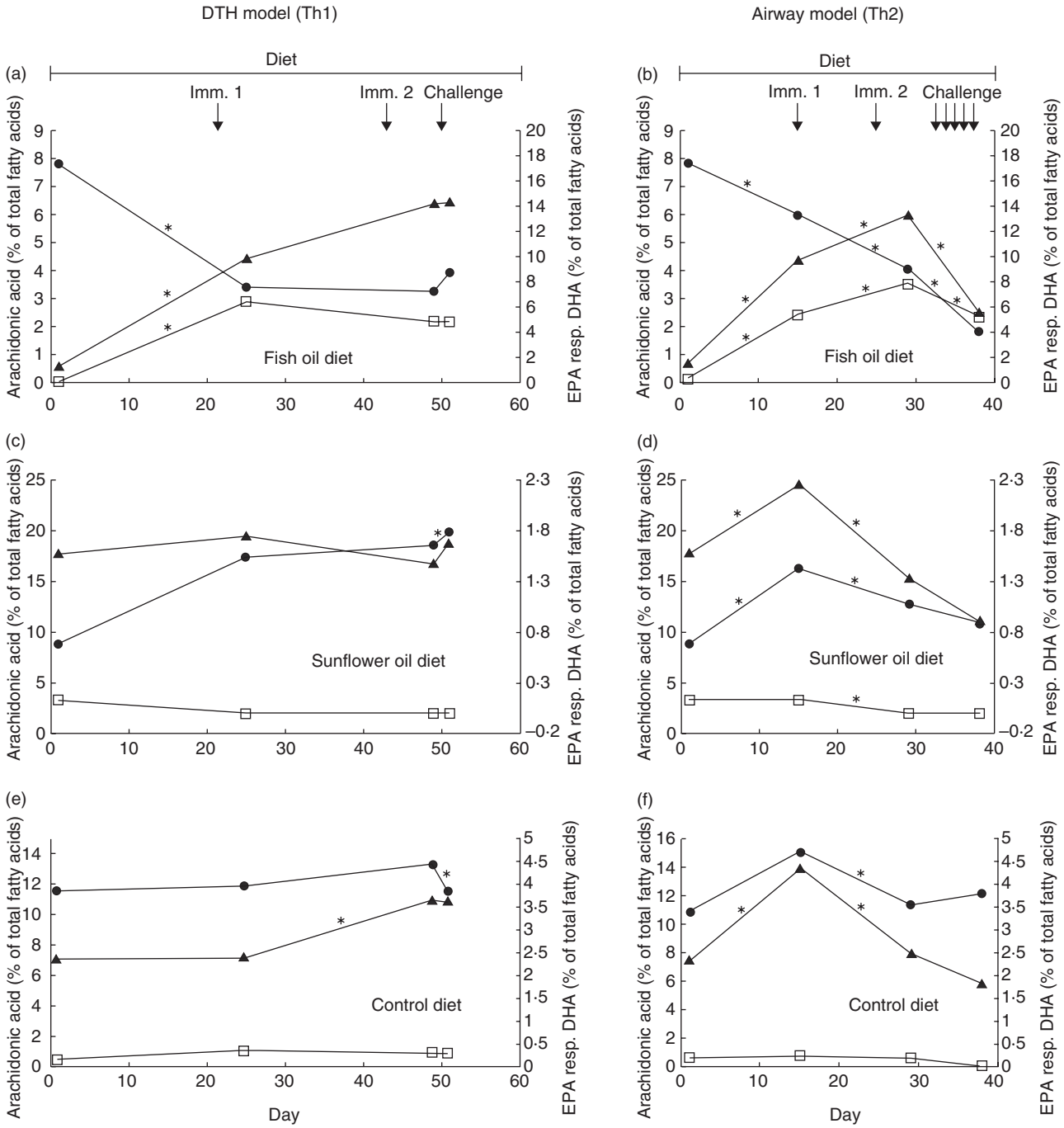

Fig. 4. Serum fatty acid levels in mice fed diets containing fish oil (a,b) or sunflower oil (c,d) supplemented or control diet (e,f). Delayed-type hypersensitivity (DTH) model: serum was collected before feeding, after one and two immunizations with ovalbumin (OVA)/Freund's adjuvant and after challenge. Airway hypersensitivity model: serum samples were collected before feeding, 2 weeks later, after two immunizations with OVA/alum and after challenge. Arachidonic acid is represented on the left *y*-axis, whereas eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are represented on the right *y*-axis. imm., immunization. $^*P < 0{\cdot}05$. ●: arachidonic acid; □: EPA; ▲: DHA acid.

and DHA in the control group (Fig. 4f) during airway challenge and subsequent inflammation. Interestingly, arachidonic acid levels also decreased significantly during airway challenge in the fish oil-fed group (Fig. 4b). A similar but non-significant reduction was seen in mice fed the sunflower oil (Fig. 4d).

The drop in serum EPA levels during the challenge phase of the Th2 model correlated positively with the serum levels of OVA-specific IgE (Fig. 5a, $r_s = 0{\cdot}48$; $P = 0{\cdot}034$). In the Th1 model, footpad swelling correlated positively with reductions in serum EPA levels during the challenge phase (Fig. 5b, $r_s = 0{\cdot}60$; $P = 0{\cdot}01$).

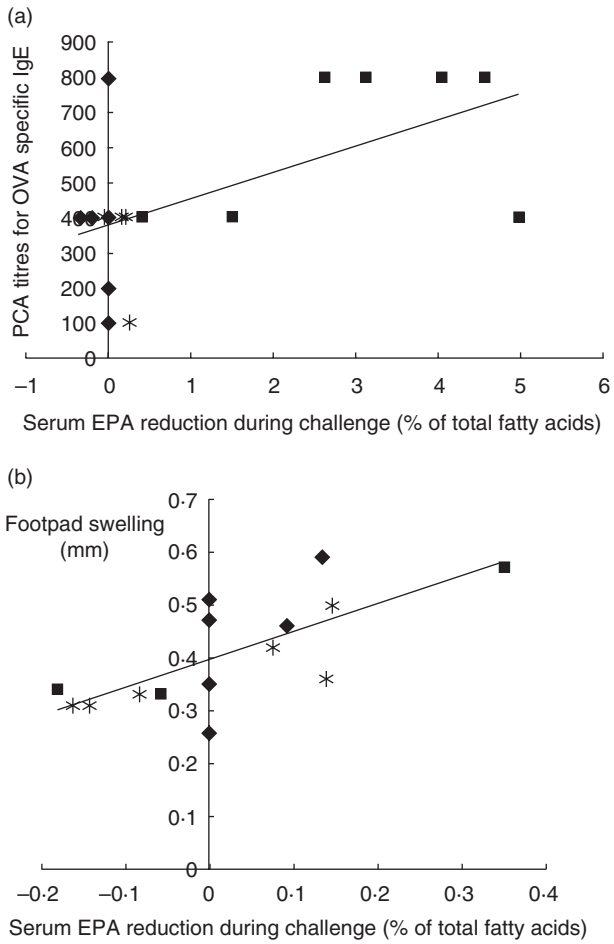

Fig. 5. (a) Airway hypersensitivity model: correlation between serum eicosapentaenoic acid (EPA) reduction during challenge and ovalbumin (OVA)-specific immunoglobulin (Ig)E ($r_s = 0{\cdot}48$; $P = 0{\cdot}034$). (b) Delayed-type hypersensitivity (DTH) model: correlation between serum EPA reduction during challenge and footpad swelling ($r_s = 0{\cdot}60$; $P = 0{\cdot}01$). ■: fish oil diet; ◆: sunflower oil diet; *: control diet.

Discussion

In the present study we investigated the effect of fatty acid supplementation on Th1 and Th2 immune reactions to a common model antigen (OVA) in mice fed either a control diet, a diet supplemented with fish oil or a diet supplemented with sunflower oil.

In the Th1 model, significantly reduced OVA-stimulated cell proliferation and production of cytokines by lymph node cells were demonstrated in the fish oil-fed group. Lymphocytes from mice fed sunflower oil also produced reduced cytokine levels than cells from mice fed the control diet. When challenged, the fish oil-fed mice showed marginally less footpad swelling than mice from the other groups. As this effect could be accounted for readily by lower prevalence and/or functional activity of Th1 memory cells, we have no evidence for any non-specific anti-inflammatory effect of fish oil in this model. However, the radically reduced antigen-induced lymphocyte proliferation and accompanying cytokine production in the fish oil-fed group confirm previous findings that a fish oil diet exerts a strong immunomodulatory (anti-Th1) effect [17,21]. The reduced levels of cytokines in the sunflower oil-fed group *versus* controls suggest that unsaturated fatty acids of the n-6 series also suppress Th1 immunity. The n-6 fatty acid arachidonic acid is a precursor of prostaglandins, which are known to counteract T cell proliferation strongly [22].

In the airway hypersensitivity model, fish oil supplementation tended to increase production of OVA-specific and total IgE antibodies and did not reduce the influx of eosinophilic granulocytes into the lungs, two prominent features of the Th2 reaction. Although the effects were moderate, our results are clearly not compatible with a protective effect against Th2-driven reactions from fish oil supplementation.

Interestingly, the most convincing effect of a fish diet on clinical allergy is reduction of atopic eczema [1–3]. Atopic eczema has a strong Th1 component; in fact, the chronic lesion is driven by Th1 cells [23]. Thus in early and acute eczema lesions, increased levels of the Th2 cytokine IL-4 are observed; later, IL-4 levels decline and levels of the Th1 cytokine IFN-γ increase [6]. These observations indicate that Th2 cells initiate atopic eczema with rapid-onset but short-lasting inflammation, whereas Th1 cells induce the chronic inflammation reaction with a later onset but a prolonged effect [7]. This biphasic pattern makes atopic eczema different from the traditional Th2 reaction observed in asthma or allergic rhinitis and conjunctivitis, which are driven by typical Th2 cytokines.

We analysed serum levels of fatty acids following the intake of test diets. Interestingly, we were able to demonstrate a profound drop in unsaturated fatty acid levels concomitant with the challenge phase and the resulting inflammatory response in the airway hypersensitivity model. The reduction was particularly prominent for levels of EPA and DHA, and EPA correlated positively and significantly with the OVA-specific IgE serum levels. This shows a considerable consumption of these fatty acids during Th2-driven inflammation. Furthermore, the results suggest that mediators formed from EPA and DHA play an active role in the Th2-driven reaction. We saw no significant decline in PUFA levels related to immunization or challenge in the DTH model, except for arachidonic acid in the control group, even though footpad swelling in individual animals correlated positively with reductions in serum EPA levels during the challenge phase. Evidently, the Th1-mediated inflammation did not consume the same amounts of fatty acids as the Th2-mediated inflammation. This could be explained by the difference in the size of the organs assessed in the two models – paws in the DTH model compared with the entire respiratory system in the airway model. Another possibility is that Th2-driven inflammation consumes large amounts of fatty acids because eosinophils are versatile producers of products

from unsaturated fatty acids [24]. Further, we observed a reduction of PUFA levels concomitant with immunization with a Th2-promoting adjuvant (alum), but not alongside immunization with a Th1-promoting adjuvant (Freund's complete adjuvant). Th1 immunity was actually accomplished by an increase in serum arachidonic acid and DHA levels after immunization. The consumption of PUFAs during the Th2- but not the Th1-sensitization phase opens the possibility that lipid mediators formed from PUFAs participate in producing the outcome of the interaction between the antigen-presenting cell and the naive T cell, in a way leading to Th2 cell maturation. The mechanisms can only be speculated upon and need further investigation. PUFAs affect gene transcription factors [25], production of prostaglandins and related mediators and affect thrombocyte activation and coagulation, processes that are linked intimately to inflammation and immunity [26].

In conclusion, our results demonstrate clearly the complexity of the immunomodulatory effects of PUFAs and point to the importance of a clear definition of the type of immune reaction involved before testing PUFA supplementation as a preventive or disease-modulatory treatment. PUFA supplementation could probably be of significance to patients suffering from Th1-mediated food allergies. However, at present we cannot draw conclusions concerning effects of PUFA supplementation on patients suffering from allergies that are complex mixtures of Th1 and Th2 immune reactions.

Acknowledgements

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Disclosure

The authors declare no financial or commercial conflicts of interest.

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